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Adenovirus-mediated transfer of low density lipoprotein receptor gene acutely accelerates cholesterol clearance in normal mice

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ABSTRACT **We have explored the use of adenovirus-mediated gene transfer to transiently elicit production of low density lipoprotein (LDL) receptors in mice. A recombinant adenovirus carrying the human LDL receptor cDNA restored LDL receptor function in receptor-deficient cultured cells. Intravenous injection of recombinant virus acutely lowered plasma cholesterol levels and increased the rate of ^{125}I-labeled LDL clearance from the circulation in normal mice. At 4 days after virus injection, the $t_{1/2}$ of plasma LDL was reduced up to 10-fold. An estimated 90% of the parenchymal cells in liver expressed the adenovirus-transferred genes as judged by immunofluorescence of LDL receptors or by β-galactosidase staining. These results demonstrate that adenovirus-mediated transfer of the LDL receptor gene provides an efficient way of augmenting LDL receptor gene function in the liver over the short term.**

The abiilty to express genes acutely in the liver provides a powerful tool for the study of metabolism and genetic metabolic disorders in intact animals. One example of such a disorder is familial hypercholesterolemia, a frequent human genetic disease that is caused by mutations in the low density lipoprotein (LDL) receptor gene (1–5). Several possible strategies to introduce the LDL receptor and other genes into cells in culture and into tissues *in vivo* have been used in the past (6–15). These experiments have included introduction of the LDL receptor gene into hepatocytes that were successfully returned to the liver (11), and they have also included intravenous administration of a plasmid containing the LDL receptor gene (16).

Recently, adenovirus-mediated gene transfer has been recognized to provide a means of high-efficiency gene transfer into a broad spectrum of eukaryotic cells (12, 17, 18) and into whole animals (6, 7, 19). In a pioneering study, adenovirus-mediated gene transfer was used to cure a rare recessive genetic disorder, ornithine transcarbamylase deficiency, in newborn mice (6). Enzymatic activity expressed from the transferred gene was sustained for up to 15 months. Similarly, the gene for human α_1-antitrypsin has been introduced into the livers of normal rats by intraportal injection (20).

In this study we have investigated the metabolic effect of adenovirus-mediated introduction of the human LDL receptor gene into the normal mouse liver. We have also assessed the efficiency of gene transfer in a quantitative way.

MATERIALS AND METHODS

Preparation of ^{125}I-Labeled LDL (^{125}I-LDL). Human plasma LDL was prepared as described and iodinated by the iodomonochloride method (21). Specific activities of ^{125}I-LDL preparations were 200 and 550 cpm per ng of protein.

Preparation of Recombinant Adenovirus. Recombinant adenoviruses (22) containing the cDNA encoding the human LDL receptor (AdCMV-LDLR) (CMV, cytomegalovirus) (23), β-galactosidase (AdCMV-βGal) (24), and firefly luciferase (AdCMV-Luc) (25) were prepared essentially as described using cotransfection of pACCMVpLpA (26) and pJM17 (27) into 293 cells (28).

Large Scale Preparation of Recombinant Adenovirus. Large scale production of recombinant adenovirus was performed in 293 cells grown either in 15-cm culture dishes or in suspension using Joklik's calcium-free minimum essential medium (GIBCO) supplemented with 10% fetal calf serum. Infected cells were lysed 48 hr postinfection with Dulbecco's phosphate-buffered saline (PBS) (GIBCO) containing 1 mM $MgCl_2$ and 0.1% Nonidet P-40. Virus-containing extracts were centrifuged at 12,000 × *g* for 10 min to remove debris before precipitation of the virus particles by addition of 0.5 vol of 20% polyethylene glycol (PEG) 8000/2.5 M NaCl and incubation on ice for 1 hr. Virus was collected by centrifugation at 12,000 × *g* for 10 min, resuspended in isotonic saline (135 mM NaCl/5 mM KCl/1 mM $MgCl_2$/10 mM Tris·HCl, pH 7.4), and dialyzed against the same buffer overnight before sterilization through a 0.22-μm filter. Alternatively, PEG-precipitated virus was further purified by CsCl density centrifugation essentially as described (29). Equivalent results were obtained with both methods of preparing virus.

^{125}I-LDL Degradation in ldlA7 Cells. Chinese hamster ovary (CHO) ldlA7 cells (30) in six-well dishes were infected with the indicated amount of recombinant virus at densities of 6×10^5 and 1.7×10^6 cells per well in 1 ml of Dulbecco's modified Eagle's medium (DMEM) containing 2% fetal calf serum. ^{125}I-LDL (4 μg/ml) in DMEM without glutamine containing 0.2% bovine serum albumin was added to the cells 60 hr after infection and incubation was continued for 6 hr. The appearance of ^{125}I-LDL degradation products in the medium was determined by measuring trichloroacetic acid-soluble radioactivity in the medium as described (21).

Immunohistochemistry. Frozen liver sections were analyzed by immunocytochemistry using polyclonal (31) and monoclonal (32) antibodies as described (31).

***Escherichia coli* β-Galactosidase and Luciferase Assays.** For β-galactosidase staining, frozen sections were fixed at room temperature in 0.5% glutaraldehyde freshly prepared in PBS for 15 min and extensively washed in PBS. β-Galactosidase activity was detected by immersing the sections into 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside (X-Gal) staining solution [35 mM $K_4Fe(CN)_6$/35 mM $K_3Fe(CN)_6$/1 mM $MgCl_2$/1 mg of X-Gal per ml (GIBCO/BRL)] for 15 hr at 37°C. Sections were lightly counterstained with eosin. The enzymatic activity of firefly luciferase was determined as described by deWet *et al.* (25).

Animal Procedures. Mice used in this study were either purchased from Harlan (BALB/c, C57BL/6) or bred in house (outbred animals) and fed ad libitum throughout the course of the experiments. Prior to virus injections and turnover studies, animals were anesthetized by intraperitoneal sodium

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Abbreviations: LDL, low density lipoprotein; CMV, cytomegalovirus; pfu, plaque-forming units.

pentobarbital (Nembutal) injection (100 μg per g of body weight). The external jugular vein was laid open by a skin incision and the indicated virus or ^{125}I-LDL was slowly (over ≈30 s) injected in a total vol of 250 μl. The wound was closed by stapling.

^{125}I-LDL in 10 mM Tris·HCl/140 mM NaCl, pH 7.5, containing 0.2% bovine serum albumin was injected 4 days after virus administration into the opposite jugular vein or into a femoral vein. Blood (50–100 μl) was obtained at the indicated intervals by retroorbital puncture from the anesthetized animals and collected into heparin-treated Pasteur pipettes. ^{125}I-LDL levels in plasma were determined by measuring trichloroacetic acid-precipitable radioactivity of a 20-μl plasma sample.

For determination of ^{125}I-LDL uptake into the different tissues, animals were killed 20 min after injection of the label. Indicated organs were removed and homogenized in PBS, and radioactivity was determined in a sample of the homogenate.

Recombinant DNA Techniques and Protein Determinations. DNA manipulations were performed essentially as described in Sambrook *et al.* (33). Protein concentrations were determined by the method of Lowry *et al.* (34). Cholesterol concentrations were determined by the cholesterol oxidase method (Boehringer Mannheim).

RESULTS

Virally Transferred LDL Receptor Is Functional in Cultured Cells. To quantify functional activity of AdCMV-LDLR, we determined the potency of the recombinant virus to confer the ability to degrade ^{125}I-LDL on CHO cells that carry a defect in their endogenous LDL receptor gene (CHO ldlA7 cells) (30). Fig. 1 shows that the amount of ^{125}I-LDL that is degraded by these cells increases in a linear fashion with the number of plaque-forming units (pfu) of AdCMV-LDLR that had been added to the culture dish. The addition of an equivalent number of AdCMV-βGal virus did not enhance degradation of the added ^{125}I-labeled ligand. The total amount of human LDL receptor protein produced by 293 cells 20 hr after infection with AdCMV-LDLR was also dependent on the amount of virus used to infect the cells (Fig. 1 *Inset*).

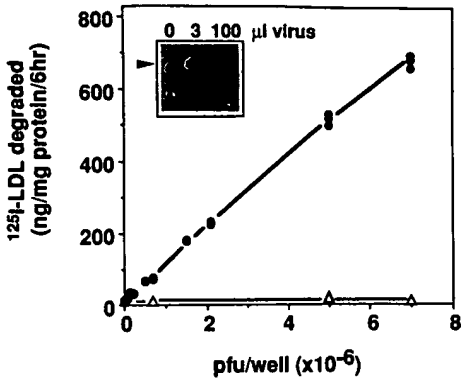

FIG. 1. AdCMV-LDLR leads to a dose-dependent increase of ^{125}I-LDL degradation in LDL receptor-defective CHO cells. LDL receptor-defective CHO ldlA7 cells were infected with the indicated number of AdCMV-LDLR (●) or AdCMV-βGal (△) as described. Degradation of ^{125}I-LDL (4 μg/ml) was measured 60 hr postinfection and normalized to the amount of protein present in each dish. Determinations were performed in triplicate and all individual data points are plotted. Some points are not resolved, as experimental variation was very small. (*Inset*) Western blot of ≈50 μg of 293 cell protein with a monoclonal antibody directed against the human LDL receptor 20 hr after mock infection (0 μl) or after infection with 3 or 100 μl of primary virus stock. Monoclonal antibody bound to LDL receptor was detected with ^{125}I-labeled rabbit anti-mouse IgG (32).

To quantify the efficiency of adenovirus-mediated gene transfer to different tissues *in vivo*, we injected ≈2 × 10^9 pfu of AdCMV-Luc into the external jugular vein of five mice. Animals were sacrificed 4 days after the injection and luciferase activity was determined in the individual tissues (Fig. 2*A*). Low luciferase activity was detected in all of the tissues examined including skeletal muscle and heart, organs that had previously been shown to be target tissues for intravenously injected recombinant adenovirus (36). However, >99% of the total enzyme activity recovered from the

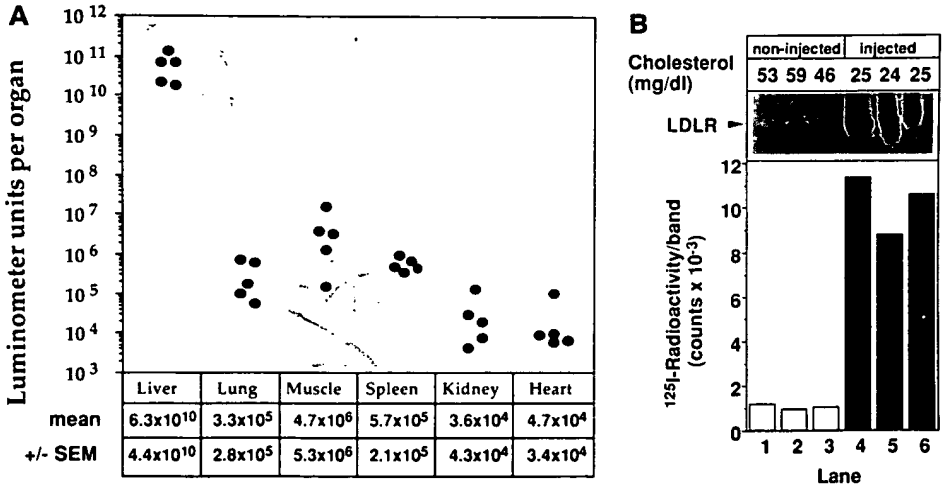

FIG. 2. (*A*) Tissue distribution of luciferase activity in AdCMV-Luc-injected animals: Preferential targeting of adenovirus to the liver. Five outbred hybrid male mice (≈6 months old; 35–45 g) were injected with ≈2 × 10^9 pfu of AdCMV-Luc into the external jugular vein. Luciferase activity in homogenates of the individual organs was determined 4 days after injection of the virus. Activities are expressed as luminometer units per total organ with the exception of muscle, where activity is expressed on a per g basis. Note that enzymatic activity is expressed on a logarithmic scale. Lower detection limit in this experiment was ≈2 × 10^3 units. (*B*) Intravenous injection of AdCMV-LDLR into normal mice leads to LDL receptor overexpression in liver. Six female C57BL/6 mice (12 weeks old; 20–25 g) were either not injected (lanes 1–3) or injected (lanes 4–6) with 2 × 10^9 pfu of AdCMV-LDLR. Four days after injection, the animals were sacrificed, and liver membranes prepared from each animal as described (35) were subjected to Western blotting (250 μg of protein per lane). Migration of the LDL receptor (LDLR) is indicated by the arrowhead. Total radioactivity present in each band was quantified by scanning the blot for 4 hr on an Ambis radioanalytic imaging system. Background (≈200 counts for each sample) was measured in a representative area of the blot and subtracted from the total counts to give the values shown.

injected mice was found in the liver. No luciferase activity was expressed in uninjected animals, which had uniform background levels regardless of the tissue examined (data not shown).

***In Vivo* Expression of Transferred Human LDL Receptor.** To assess whether the recombinant virus was efficient in causing a physiologically relevant increase of LDL receptor activity in the liver, the primary site of lipoprotein catabolism (1), we injected three C57BL/6 female mice with $\approx 2 \times 10^9$ pfu of AdCMV-LDLR into the external jugular vein. Four days later, hepatic expression of the transferred LDL receptor was evaluated by Western blotting. Expression of LDL receptors in the livers of virus-injected animals was increased ≈10-fold over that observed in uninjected control mice (Fig. 2*B*), assuming equal reactivity of the antibody with human and mouse receptors. Virus-injected animals also had significantly lower plasma cholesterol levels when compared with noninjected controls, indicating that the LDL receptor cDNA contained in the virus was transcribed and translated into biologically functional receptors. We next sought to determine the expression pattern of the virally transferred genes for human LDL receptor and *E. coli* β-galactosidase in the livers of injected mice. For this purpose, liver sections of animals that had been injected with either AdCMV-LDLR or AdCMV-βGal were examined by immunofluorescence analysis (Fig. 3 *A–D*) or by histochemical staining for β-galactosidase activity (Fig. 3 *E* and *F*). LDL receptor was expressed only in the livers of animals that had been injected with AdCMV-LDLR (Fig. 3 *A*, *B*, and *D*) and was absent in animals that had been injected with AdCMV-βGal (Fig. 3*C*). Likewise, a similarly high percentage of liver cells of animals that had been injected with the adenovirus construct carrying the β-galactosidase gene were reactive upon histochemical examination for this enzyme (Fig. 3*E*), while β-galactosidase activity was completely absent from the livers of mice injected with AdCMV-LDLR (Fig. 3*F*).

Increased Catabolic Rate of ^{125}I-LDL. To further quantitate the effect of the virus-mediated transfer of exogenous LDL receptor cDNA, we examined the rate of ^{125}I-LDL turnover and steady-state cholesterol levels in animals injected with

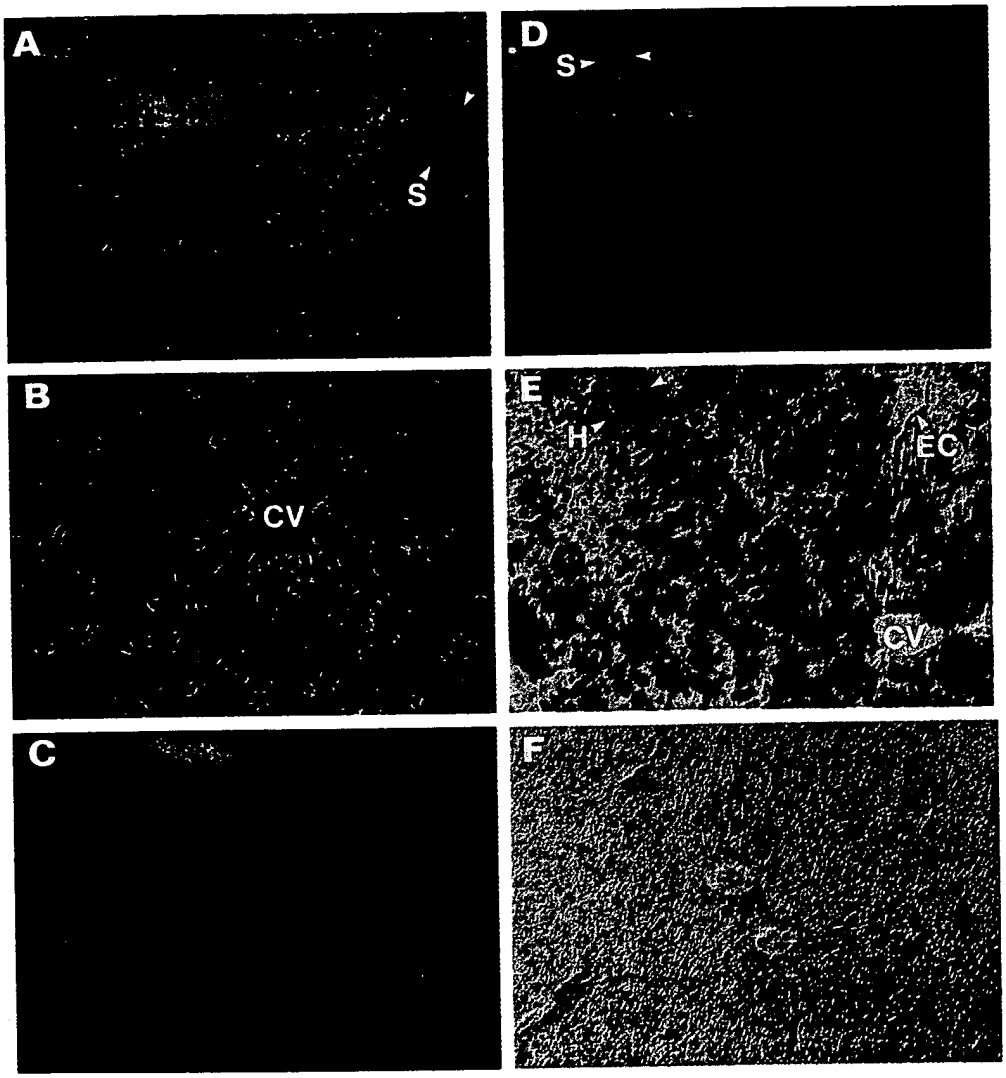

FIG. 3. Immunohistochemical analysis of human LDL receptor expression and histochemical detection of β-galactosidase activity in the livers of mice injected with AdCMV-LDLR or AdCMV-βGal. Female BALB/c mice (≈12 weeks old; 20–25 g) were injected with either 3.5 $\times 10^9$ (*A*, *D*, and *F*) or 2×10^9 (*B*) pfu of AdCMV-LDLR or with 2×10^9 (*C* and *E*) pfu of AdCMV-βGal. Expression of LDL receptor in livers of animals was detected with either a polyclonal rabbit IgG (*A–C*) or a mouse monoclonal IgG (*D*) that reacts specifically with the human receptor. Specific staining is only present in the livers of animals injected with AdCMV-LDLR and is absent in the AdCMV-βGal-injected mouse. Up to an estimated 90% of the liver cells are expressing human LDL receptor, which shows the typical polarized expression pattern (37). Sinusoids (S) (*A* and *D*) that have been sectioned along the longitudinal axis are indicated by arrowheads. Expression of β-galactosidase activity (*E*) is found predominantly in the nuclei of hepatocytes (H) that are arranged in a typical columnar array (arrowheads in *E*). Nuclei of endothelial cells (EC) are stained less intensely. No β-galactosidase activity is found in mice injected with AdCMV-LDLR (*F*). CV, central vein. (×28.)

AdCMV-LDLR versus mice that had received AdCMV-βGal. Fig. 4 shows the results of four separate clearance studies performed with two different virus preparations. ^{125}I-LDL was removed from plasma slowly by the control animals injected with AdCMV-βGal and the clearance rate ($t_{1/2}$, ≈5 hr) was indistinguishable from that previously observed in normal mice (38). In contrast, AdCMV-LDLR significantly accelerated removal of the radiolabeled ligand from the circulation of the animals. As a rule, the animals that cleared the ^{125}I-labeled ligand most efficiently also had the lowest steady-state plasma cholesterol levels 4 days after virus administration (Fig. 4 and Table 1). The rate of ^{125}I-LDL clearance was dose dependent and proportional to the amount of pfu of AdCMV-LDLR that had been injected into the mice and was up to 10-fold greater than the rate observed in either normal (38) or AdCMV-βGal-injected animals (this study).

The liver was the only organ in AdCMV-LDLR-injected mice that showed a significant increase in ^{125}I-LDL radioactivity versus control animals when the absolute tissue uptake of the labeled ligand was measured 20 min after injection (Table 1). Up to 45% of the injected dose was recovered in the livers of AdCMV-LDLR-injected mice compared to 13–15% that had accumulated in the controls. Thus, even without compensating for nonspecific trapping of the tracer, significantly more ^{125}I-LDL was taken up into the livers of AdCMV-LDLR-injected animals, in agreement with the results obtained for AdCMV-Luc (Fig. 2*A*).

DISCUSSION

We have explored the feasibility of using replication-defective adenovirus particles to transfer the LDL receptor gene into the liver. Following a single peripheral intravenous injection, a high proportion of hepatic parenchymal cells

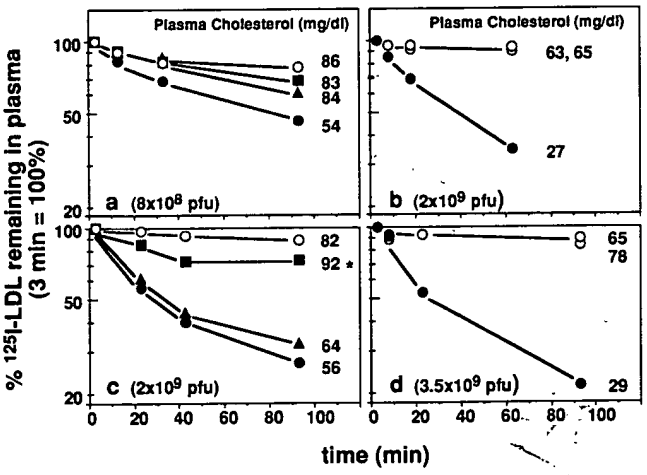

FIG. 4. ^{125}I-LDL clearance from plasma is accelerated in mice injected with AdCMV-LDLR. Female BALB/c mice (≈12 weeks old; 20–25 g) were injected with the indicated amount of AdCMV-LDLR (solid symbols) or with a fixed amount of AdCMV-βGal (2×10^9 pfu; open circles). Four days after virus administration, animals were injected with 15 μg of ^{125}I-LDL. Blood samples were analyzed at the indicated times as described and the radioactivity remaining in plasma was plotted as a percentage of the activity present 3 min after injection of the labeled ligand. Four separate experiments were performed. Individual clearance curves for each animal are shown. Steady-state plasma cholesterol levels (mg/dl) of each animal are indicated next to the last time point of the clearance curve. Asterisk denotes a mouse that showed shock symptoms during the clearance experiment and died shortly after the last time point was taken. This animal ceased to clear ^{125}I-LDL between 40 and 90 min. Hepatic circulation was presumably shut down as a result of circulatory shock.

Table 1. Tissue uptake of ^{125}I-LDL 20 min after injection

	% of injected dose					
	LDLR			β-Gal		
Liver	44.6	44.4	21.9	12.9	15.2	14.7
Kidney	1.1	2.2	2.5	2.2	3.4	2.9
Lung	0.9	1.4	1.8	1.7	1.7	1.4
Spleen	0.5	0.6	0.7	1.1	1.2	1.1
Heart	0.3	0.7	0.7	1.2	1.0	0.9
Plasma cholesterol, mg/dl	42	63	84	81	102	86

Female BALB/c mice (12 weeks old; 20–25 g) were injected with either 2×10^9 pfu of AdCMV-LDLR or the same amount of AdCMV-βGal. Four days after virus administration, the animals were injected with 7.5 μg of ^{125}I-LDL and killed 20 min later. Organs were quickly removed and homogenized in PBS; radioactivity was determined in a sample of the homogenate. Total radioactivity recovered from the individual organs is shown as a percentage of injected dose (4×10^6 dpm). No correction has been made for nonspecific trapping of the ^{125}I-labeled tracer. Plasma cholesterol levels of individual animals are shown in the bottom line.

were infected with the recombinant virus and efficiently expressed foreign genes were harbored within the adenovirus genome. Expression of the human LDL receptor gene resulted in plasma cholesterol levels that were lower than those measured in animals that had been injected with a virus carrying the β-galactosidase gene. Likewise, clearance of ^{125}I-LDL from the circulation was up to 10-fold accelerated in mice injected with AdCMV-LDLR. This is consistent with the ≈10-fold increase of LDL receptor immunoreactivity in the livers of injected mice. Adenovirus-mediated gene transfer therefore can provide strong transient expression of an exogenous LDL receptor gene in the liver, predominantly in the hepatocytes. The liver is the primary target after peripheral intravenous injection of recombinant adenovirus as shown quantitatively in this study and was previously observed by Stratford-Perricaudet *et al.* (36).

We have not yet explored other important questions that are essential to evaluate the potential suitability of the adenovirus system for the treatment of familial hypercholesterolemia or other inborn errors of metabolism that manifest themselves in the liver. These include the choice of the promoter driving the exogenous gene, safety concerns connected with the use of a human virus (20), and the efficiency of virus-mediated gene transfer in patients or animals expressing neutralizing antibodies to adenovirus. It is also currently not clear for how long the adenovirus genome will persist in various tissues and whether persistence of gene expression will be longer in tissues that turn over slowly versus those that regenerate more rapidly (35).

A pathological pneumonia-like response after intranasal inoculation of mice with type 5 adenovirus independent of viral replication has been reported (40). In our study, we have also observed variable degrees of lymphocytic infiltrations in the livers of virus-injected mice (data not shown). The extent of the inflammatory reaction appeared to be proportional to the amount of administered virus and was paralleled by an increase of liver marker enzymes in the plasma. To assess the potential therapeutic use of adenoviral LDL receptor gene transfer into the liver, further investigation of cytotoxic side effects caused by the virus will be required.

In the present experiments, the LDL receptor cDNA was driven by the strong CMV promoter. Overexpression of LDL receptors does not appear to have any obvious harmful effects in transgenic mice (36) and usage of strong endogenous promoters (like the apolipoprotein AI promoter) might be preferable and could potentially improve long-term expression of the transferred gene. This question will have to be addressed in the only animal model available for this disease to date, the Watanabe heritable hyperlipidemic rabbit (39), or

possibly in mice in which the LDL receptor gene has been eliminated by targeted disruption.

Irrespective of whether it will prove to be a feasible approach for somatic gene therapy adenovirus-mediated gene transfer is a powerful method that allows at least transiently a high level of expression of a functional foreign gene in the liver of an intact animal. In many cases this method may obviate the need to create transgenic animals.

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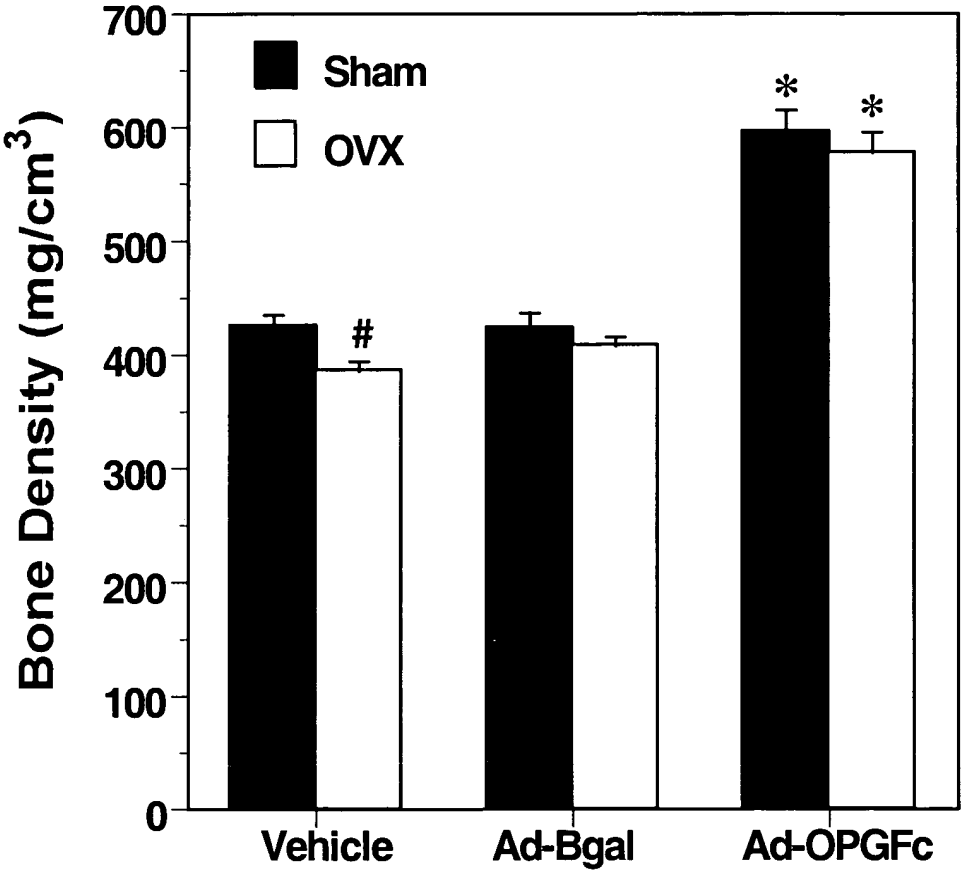
ATTACHMENT NO. 6

BONE MINERAL DENSITY IN THE TIBIAL METAPHYSIS
OF SHAM-OPERATED AND OVARIECTOMIZED (OVX) MICE

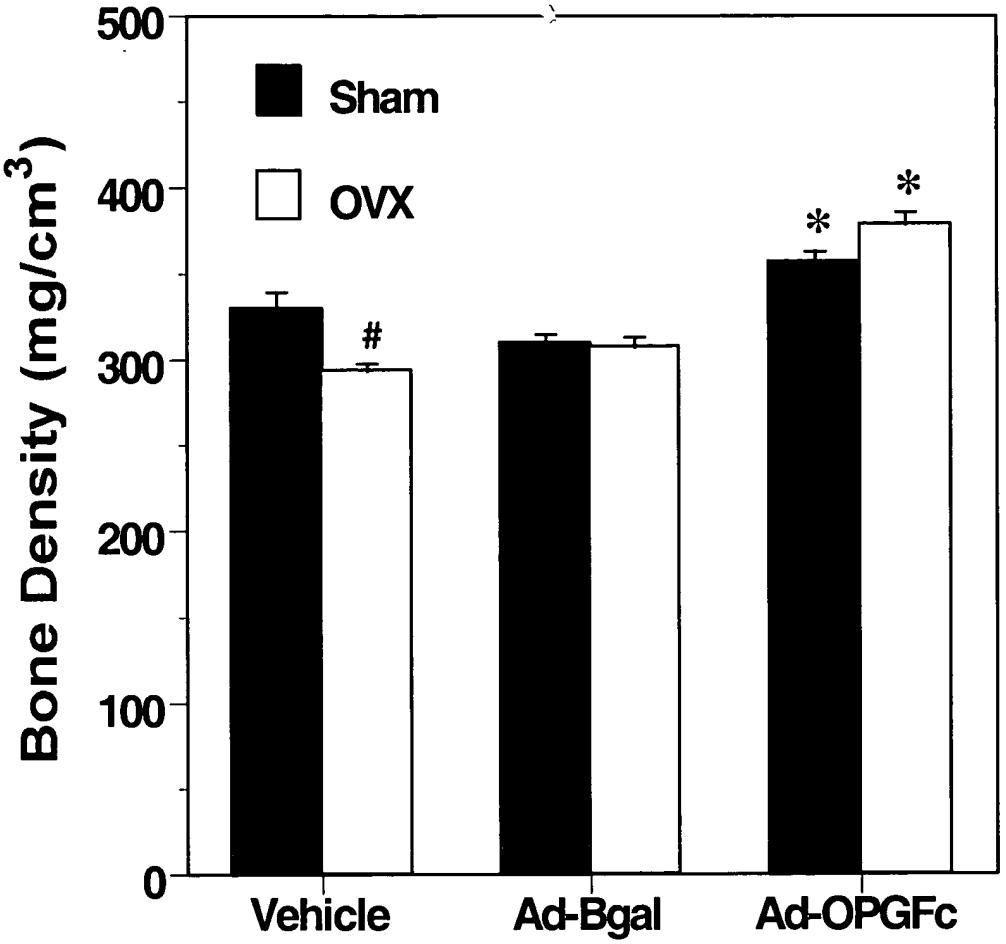


Ovariectomized mice are significantly different from sham-operated mice receiving the same treatment ($p < 0.05$)

* Ovariectomized and sham-operated mice receiving Ad-OPGFc are significantly different from sham-operated mice receiving vehicle ($p<0.001$)

ATTACHMENT NO. 7

BONE MINERAL DENSITY IN THE FIFTH LUMBAR VERTEBRA OF SHAM OPERATED AND OVARIECTOMIZED (OVX) MICE



Ovariectomized mice are significantly different from sham-operated mice receiving the same treatment, ($p < 0.05$)

* Ovariectomized and sham-operated mice receiving Ad-OPGFc are significantly different from sham-operated mice receiving vehicle ($p < 0.001$)